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DIAGNOSTICS AND MOLECULAR EPIDEMIOLOGY OF *cpe*-POSITIVE *CLOSTRIDIUM PERFRINGENS* TYPE A

Annamari Heikinheimo

ACADEMIC DISSERTATION

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ABBREVIATIONS

a_w	water activity
AAD	antibiotic-associated diarrhea
AFLP	amplified fragment length polymorphism
AOAC	Association of Official Analytical Chemists
ATCC	American Type Culture Collection
BA	blood agar
bp	base pair
CCUG	Culture Collection of the University of Gothenburg
CH	colony hybridization
CMM	cooked meat medium
cpa	gene encoding *Clostridium perfringens* alpha toxin
cpb	gene encoding *Clostridium perfringens* beta toxin
cpe	gene encoding *Clostridium perfringens* enterotoxin
CPE	*Clostridium perfringens* enterotoxin
dcm	gene encoding cytosine methylase
DIG	digoxigenin
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate
ELISA	enzyme-linked immunosorbent assay
etx	gene encoding *Clostridium perfringens* epsilon toxin
FRET	fluorescence resonance energy transfer
GC	guanine and cytosine
GI	gastrointestinal
HGMF	hydrophobic grid membrane filter
HGT	horizontal gene transfer
IMM	iron milk medium
iA	gene encoding *Clostridium perfringens* iota toxin
IS	insertion sequence
ISO	International Standardization Organization

kb	kilobase
LS	lactose-sulfite broth
Mb	megabase
MEM	minimum essential media
MGE	mobile genetic element
MLET	multilocus enzyme typing
MLST	multilocus sequence typing
MLVNTR	multilocus variable-number tandem repeat analysis
MPN	most probable number
mRNA	messenger ribonucleic acid
MRP	macrorestriction pattern
NaCl	sodium chloride
Na_2HPO_4	sodium hydrogen phosphate
$NaNO_3$	sodium nitrate
NCFA	Nordic Committee on Food Analysis
NCTC	National Collection of Type Cultures
OPSP	oleandomycin polymyxin sulfadiazine-perfringens medium
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PIV	tris-NaCl buffer
ppm	parts per million
RAPD	randomly amplified polymorphic DNA
rep-PCR	repetitive element PCR
RFLP	restricted fragment length polymorphism
RPLA	reversed passive latex agglutination
SD	sporadic diarrhea
SDS	sodium dodecyl sulfate
SFP	Shahidi Ferguson Perfringens agar
SIDS	sudden infant death syndrome, cot death
SPS	sulfite polymyxin sulfadiazine medium
TSC	tryptose sulfite cycloserine medium
U	unit
Vero cell	African green monkey kidney cell

ABSTRACT

Clostridium perfringens enterotoxin (CPE)- producing *C. perfringens* type A is considered one of the most common causes of food poisonings in the industrialized world. CPE-producing *C. perfringens* type A is also involved in antibiotic-associated diarrhea and sporadic diarrhea in humans as well as in animals. *C. perfringens* is widely dispersed, but enterotoxin gene-carrying (*cpe*-positive) isolates are rarely found in nature. Thus, not much is known about reservoirs or transmission routes of this pathogen. This work aimed to improve diagnostics of *cpe*-positive *C. perfringens* by developing a method for the detection and isolation of *cpe*-positive *C. perfringens* type A. Other objectives were to improve identification and typing of *C. perfringens*. With novel assays, molecular epidemiology of *cpe*-positive *C. perfringens* type A was further investigated.

Development of the hydrophobic grid membrane filter-colony hybridization (HGMF-CH) method for the detection and isolation of *cpe*-positive *C. perfringens* from feces has provided an effective tool since none of the previously reported detection methods have facilitated the isolation of *cpe*-positive *C. perfringens*. The main advantage of the HGMF-CH method is that it allows the detection and isolation of *cpe*-positive colonies among large numbers of *cpe*-negative isolates. Thus, the method is unaffected by the presence of *cpe*-negative *C. perfringens* in samples, a frequent problem when screening *cpe*-positive *C. perfringens* from nature. Use of the HGMF-CH method in a subsequent study proved that the method is adequate for the detection and isolation of *cpe*-positive *C. perfringens* from fecal samples. By initially screening samples for the presence of *cpe* and by choosing polymerase chain reaction (PCR)-positive samples for HGMF-CH, costs are reduced and the method is rendered even more effective.

To obtain a tool for toxinotyping *C. perfringens* isolates and determining the presence of *cpe* in these isolates, a multiplex PCR assay was established. The novel assay was established by designing new *cpa* primers and combining them with *cpb, etx, iA*, and *cpe* primers from a previously published protocol. The novel assay amplified all PCR products simultaneously and was used for the investigation of 118 *C. perfringens* isolates. The assay was also used in subsequent studies for toxinotyping and determining the presence of *cpe* in *C. perfringens* isolates.

The amplified fragment length polymorphism (AFLP) protocol provides a highly reproducible, easy-to-perform, and relatively fast method for the identification and DNA fingerprinting of *C. perfringens*. This assay overcomes the problems of extracellular DNase production, which are occasionally present with other typing methods.

When investigating the presence of *cpe*-positive *C. perfringens* in the feces of healthy food-handlers, the organism was carried by 18% of the individuals. The presence of strains representing all previously identified genotypes (plasmid-borne IS*1151*-*cpe*, plasmid-borne IS*1470*-like-*cpe*, and chromosomal IS*1470*-*cpe*) as well as strains carrying previously unrecognized genetic arrangements attached to *cpe* shows that various different subpopulations of *cpe*-positive *C. perfringens* type A occur in the human gastrointestinal tract and that the healthy human is a rich reservoir for *cpe*-positive *C. perfringens* type A. Additionally, pulsed-field gel electrophoresis (PFGE) typing of the strains revealed a loss or acquisition of the *cpe* plasmid by *C. perfringens* strains. Thus, our findings support the theory of the movable nature of *cpe.* Overall, the common occurrence of *cpe*-positive *C. perfringens* type A in the feces of healthy food-handlers and the full capacity of these strains to produce CPE indicate that humans handling food should be considered a possible source of contamination of food.

By showing that 25% of European *C. perfringens* type A food poisoning outbreaks are caused by strains carrying plasmid-borne *cpe*, we reveal that the generally accepted relationship between *cpe* genotype and CPE-associated gastrointestinal diseases is not straightforward and that plasmid-borne *cpe* is a common cause of food poisonings.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by Roman numerals I-V.

I Heikinheimo, A., Lindström, M., and Korkeala, H. 2004. Enumeration and isolation of *cpe*-positive *Clostridium perfringens* spores from feces. *J Clin Microbiol* 42: 3992-3997.

II Heikinheimo, A., and Korkeala, H. 2005. Multiplex PCR assay for toxinotyping *Clostridium perfringens* isolates obtained from Finnish broiler chickens. *Lett Appl Microbiol* 40: 407-411.

III Keto-Timonen, R., Heikinheimo, A., Eerola, E., and Korkeala, H. 2006. Identification of *Clostridium* species and DNA fingerprinting of *Clostridium perfringens* by amplified fragment length polymorphism analysis. *J Clin Microbiol* 44: 4057-4065.

IV Heikinheimo, A., Lindström, M., Granum, P.E., and Korkeala, H. 2006. Humans as reservoir for enterotoxin gene-carrying *Clostridium perfringens* type A. *Emerg Infect Dis* 12: 1724-1729.

V Lahti, P., Heikinheimo, A., Johansson, T., and Korkeala, H. 2008. *Clostridium perfringens* type A isolates carrying plasmid-borne enterotoxin gene (genotypes IS*1151*-*cpe* or IS*1470*-like-*cpe*) are a common cause of food poisonings. *J Clin Microbiol* 46: 371-373.

1. INTRODUCTION

Clostridium perfringens is thought to be the most widely dispersed pathogen on earth (Canard *et al*. 1992). The presence of *C. perfringens* DNA fragments in the colon of "Tyrolean Iceman", the mummy discovered in an Alpine glacier, is evidence that *C. perfringens* and humans have coexisted for at least 5000 years (Cano 2000). However, the first report of *C. perfringens* dates back to 1891, when Doctor Welch and his colleagues found a previously unknown bacterium forming gas bubbles in an autopsied patient's blood vessels and christened the bacterium *Bacillus aerogenes capsulatus* (Welch and Nuttall 1892). The organism became known as *Bacillus welchii*. Several other names, *i.e. Bacillus enteritidis sporogenes*, *Bacillus perfringens*, *Bacterium welchii*, and *Clostridium welchii*, were also used (Andrewes 1899, Cato *et al*. 1986). During World War I, the organism was recognized as an important cause of gas gangrene in battlefield injuries, killing hundreds of thousands of soldiers (Shimizu *et al*. 2002). Later, the organism became known as *Clostridium perfringens* (i.e. a small spindle, "breaking through") (Skerman *et al*. 1980).

Today, *C. perfringens* has a notorious reputation for possessing an extremely wide toxin arsenal, with over 15 toxins. The organism is therefore capable of producing different pathological pictures and causing numerous different histotoxic and enteric diseases in both humans and animals (Smedley *et al*. 2004).

C. perfringens type A was first identified as a cause of food poisoning in the 1940s (Knox and MacDonald 1943, McClung 1945). *C. perfringens* type A food poisoning ranks annually as one of the most common food poisonings in the industrialized world (Mead *et al*. 1999, Adak *et al*. 2002, Hatakka and Pakkala 2003). In addition to food poisoning, *Clostridium perfringens* enterotoxin (CPE)-producing *C. perfringens* type A causes antibiotic-associated diarrhea and sporadic diarrhea in humans and animals.

The ubiquitous distribution of *C. perfringens* has earlier been considered a logical explanation for the common occurrence of *C. perfringens* type A food poisonings. Therefore, all *C. perfringens* isolates were regarded as potential causative agents for *C. perfringens* type A food poisonings. However, currently we know that only a small minority, less than 5% of global *C. perfringens* isolates produce CPE and are thus capable of causing food poisonings

(Smedley *et al*. 2004). Recent findings of the variable loci (plasmid vs. chromosome) and different genetic arrangements adjacent to *cpe* have shown that *cpe*-positive isolates form in different subpopulations. These subpopulations are responsible for different CPE-associated diseases, i.e. chromosomal *cpe*-bearing strains are involved in food poisonings, whereas strains carrying *cpe* in the plasmid are typical in antibiotic-associated or sporadic diarrhea cases as well as in animal diarrheas (Cornillot *et al*. 1995, Katayama *et al*. 1996, Collie and McClane 1998, Sparks *et al*. 2001). This indicates that different subpopulations of *cpe*-positive *C. perfringens* may have different epidemiologies. However, not much is known about the molecular epidemiology of *cpe*-positive *C. perfringens*, and the reservoirs and transmission routes have remained unidentified. This is partly due to methodological problems since conventional culture methods do not distinguish *cpe*-positive *C. perfringens* from *cpe*-negative *C. perfringens*, which usually dominate in natural samples.

This work aimed to develop a specific method for the detection and isolation of *cpe*-positive *C. perfringens* and to identify potential reservoirs for *cpe*-positive *C. perfringens* by using this method. The epidemiology of different genotypes of *cpe*-positive *C. perfringens* in food poisoning outbreaks was also elucidated. A further objective was to improve diagnostics of *C. perfringens* by harnessing amplified fragment length polymorphism (AFLP) as well as multiplex polymerase chain reaction (PCR) in typing and identifying *C. perfringens* isolates.

2. REVIEW OF THE LITERATURE

2.1 *Clostridium perfringens*

2.1.1 Classification and characteristics

Clostridium perfringens is a Gram-positive, catalase-negative, nonmotile, endospore-forming bacterium with a cell size of 0.6-2.4 × 1.3-19.0 µm (Cato *et al*. 1986). The spores are subterminal and oval. *C. perfringens* is a typical member of the genus *Clostridium*, requiring anaerobic conditions for growth. However, contrary to other clostridia, *C. perfringens* tolerates moderate exposure to air and requires only a relatively modest reduction in oxidation-reduction potential (E_h) for growth (Cato *et al*. 1986). *C. perfringens* is known as the most rapidly multiplying organism, with some strains possessing less than a 10-minute doubling time (Willardsen *et al*. 1978, Willardsen *et al*. 1979). The organism is divided into five types (A through E) according to the production of the major lethal toxins alpha, beta, epsilon, and iota (Brooks *et al*. 1957, Sterne and Warrack 1964) (Table 1).

Table 1. Typing of *Clostridium perfringens*.

Type	Toxin(s) produced			
	Alpha	Beta	Epsilon	Iota
A	+	-	-	-
B	+	+	+	-
C	+	+	-	-
D	+	-	+	-
E	+	-	-	+

C. perfringens was the first Gram-positive bacterium for which a complete genome map was constructed by using pulsed-field gel electrophoresis (PFGE) (Canard and Cole 1989). The

genome size of *C. perfringens* varies between 3.0 and 3.7 Mb (Rood 1997, Shimizu *et al*. 2001) and the GC content of DNA is 24-27% (Cato *et al*. 1986). Currently, the entire genome sequence of three different *C. perfringens* strains has been published (Shimizu *et al*. 2002, Myers *et al*. 2006). Comparison of sequences has revealed an unexpected degree of genomic variability between *C. perfringens* strains, with discrete islands containing genes coding for mobile elements, metabolic capabilities, extracellular capsules, toxins, and other enzymes, enabling *C. perfringens* to adapt to different environmental conditions and to possess different disease phenotypes and virulence characteristics (Myers *et al*. 2006).

2.1.2 Microbial ecology

C. perfringens is referred to as the most widely distributed pathogenic microorganism in nature (Cato *et al*. 1986). Its principal habitats are soil and the intestinal contents of humans and animals (Smith 1975, Yamagishi *et al*. 1976, Brant *et al*. 1978). *C. perfringens* is also present in marine sediments and sewage (Smith 1975, Lisle *et al*. 2004). Type A predominates in the environment and human intestines, whereas types B-E are usually carried by animals (Smith 1975). In potable water, the presence of *C. perfringens* is often used as an indicator of long-term fecal contamination (Edberg *et al*. 1997, Hörman *et al*. 2004). The organism also gains easy access to different foods and spices (Ladiges *et al*. 1974, Foster *et al*. 1977, Smart *et al*. 1979, Aguilera *et al*. 2005). Growth characteristics of *C. perfringens* are described in Table 2.

Table 2. Growth characteristics of *Clostridium perfringens.*

	Minimum	Optimum	Maximum	Reference
Temperature (ºC)	10-12	43-47	50-55	(Labbé 2000, Li and McClane 2006a)
pH	5.0-5.5	6.0-7.2	8.0-9.0	(Labbé 2000)
NaCl (%)	-	5.0-8.0[a]	-	(Labbé 2000, Li and McClane 2006a)
$NaNO_3$ (ppm)	-	60-2560[a]	-	(Labbé 2000, Li and McClane 2006a)
Water activity (a_w)	0.93	0.95-0.96	0.97	(Labbé 2000)

[a] Concentration/amount limiting the growth of *Clostridium perfringens* in different foods.

2.1.3 Diseases caused by *Clostridium perfringens*

Due to the remarkably wide toxin arsenal of more than 15 toxins, *C. perfringens* is able to cause several different enteric and histotoxic diseases in both humans and animals (Table 3). The nomination of *C. perfringens* epsilon toxin to the U.S. bioterrorism selected agent list illustrates the potency of *C. perfringens* toxins (Khan *et al*. 2000). The organism is divided into five types (A through E) according to the production of the major toxins alpha, beta, epsilon, and iota (Table 1). In addition, *C. perfringens* isolates may produce other toxins such as enterotoxin (CPE) and beta2 toxin (Gibert *et al*. 1997, Smedley *et al*. 2004). The pathogenicity of *C. perfringens* is attributed to different types (Brooks *et al*. 1957, Sterne and Warrack 1964) (Table 3). However, rather than being a function of a single toxin, the virulence of different *C. perfringens* isolates is considered a multifactorial trait, with different determinants contributing to adaptation of the organism to its niche and to producing the pathological picture (Canard *et al*. 1992, Miyamoto *et al*. 2006, Sawires and Songer 2006, Sayeed *et al.* 2007). The virulence of *C. perfringens* has not yet been fully elucidated.

Table 3. Diseases associated with *Clostridium perfringens* (Hatheway 1990, Songer 1996, Petit *et al*. 1999, Smedley *et al*. 2004, Fisher 2006).

Type	Disease(s)	
	Humans	Animals
A	Food poisoning[a] Antibiotic-associated diarrhea[a] Sporadic diarrhea[a] Sudden infant death syndrome[a] Gas gangrene Acute gastric dilatation Necrotic enteritis (infants)	Enterotoxemia (various animals) Necrotic enteritis (fowl) Intestinal clostridiosis (equine) Acute gastric dilatation (various animals) Enteritis (various animals)[a]
B		Enterotoxemia (sheep, goat, guinea pig) Dysentery (lamb)
C	Enteritis necroticans (i.e. Darmbrand or pigbel)	Enterotoxemia (lamb and pig) Struck (sheep) Necrotic enteritis (fowl) Enteritis (porcine)
D		Enterotoxemia (sheep) Pulpy kidney disease (lamb)
E		Enterotoxemia (calf, guinea pig, rabbit) Dysentery (lamb)

[a] Enterotoxin-producing strains associated with the disease.

2.2 *Clostridium perfringens* enterotoxin (CPE)

2.2.1 Characteristics of CPE

When *C. perfringens* was discovered as a cause of food poisonings, the strains were proposed to produce an enterotoxin (McClung 1945). At a later date, this toxin, characterized to be a 35–kDa single polypeptide with an isoelectric point of 4.3, was named *C. perfringens* enterotoxin (CPE) (Hauschild and Hilsheimer 1971, Stark and Duncan 1972). CPE is stabile at a pH between 5 and 12 and is inactivated with pronase, but not with trypsin, lipase, or amylase (Duncan and Strong 1969). The enteric pathogenicity of CPE has been evidenced in human volunteer feeding studies, showing that purified toxin causes diarrhea and cramping (Skjelkvale and Uemura 1977). Animal model studies with isogenic enterotoxin gene mutant have further confirmed Koch's molecular postulates about the requirement of CPE expression to cause gastrointestinal (GI) effects (Sarker *et al*. 1999).

2.2.2 Enterotoxin gene (*cpe*)

The gene coding for enterotoxin (*cpe*) has been cloned and sequenced in fragments (Hanna *et al*. 1989, Iwanejko *et al*. 1989, Van Damme-Jongsten *et al*. 1989) and in its entity (Czeczulin *et al*. 1993). *cpe*-carrying (*cpe*-positive) isolates possess a single copy of *cpe* (Cornillot *et al*. 1995). The gene is homologous in all *cpe*-positive *C. perfringens* strains studied, but 45-bp insertions have been found in the *cpe* promoter region in some strains (Melville *et al*. 1994). No considerable DNA sequence homology with other organisms has been observed (Petit *et al*. 1999). Although in CPE-associated diseases *C. perfringens* isolates usually represent type A, *cpe* can be detected in *C. perfringens* isolates representing any type (A-E). Not all isolates are, however capable of expressing the gene product. *cpe*-positive *C. perfringens* type E isolates, for example, possess mutations in an open reading frame (ORF), promoter(s), and the ribosome binding site of their *cpe* sequences and therefore do not produce CPE (Billington *et al*. 1998). However, in CPE-associated diseases, the isolate usually represents type A, perhaps because isolates of type A account for more than 95% of global *C. perfringens* isolates (McClane 2001).

cpe-positive *C. perfringens* type A strains possess *cpe* in either a chromosome or a large plasmid (Canard *et al*. 1992, Cornillot *et al*. 1995) (Fig. 1). Chromosomal *cpe*-bearing strains are involved in food poisonings, whereas strains carrying *cpe* in a plasmid are typically present in antibiotic-associated or sporadic diarrhea cases as well as in animal diarrheas (Cornillot *et al*. 1995, Katayama *et al*. 1996, Collie and McClane 1998, Sparks *et al*. 2001). In both cases, insertion sequence (IS) elements have been found adjacent to *cpe,* indicating that *cpe* is movable (Brynestad *et al*. 1994, Cornillot *et al*. 1995, Brynestad 1997, Brynestad *et al*. 1997, Brynestad *et al*. 1999, Miyamoto *et al*. 2002). In strains with *cpe* in the chromosome (chromosomal *cpe* strains), *cpe* is located on a 6.3-kb transposable element, named Tn*5565* (Brynestad *et al*. 1997, Brynestad *et al*. 1999). This apparent transposon includes two copies of IS*1470* on both sides (up- and downstream) of *cpe* (Brynestad *et al*. 1997) and may have circular intermediate forms (Brynestad *et al*. 1999). A few papers have reported a loss of *cpe* from chromosomal strains under laboratory conditions, but the actual movement of Tn*5565* has not been demonstrated (Collie and McClane 1998, Ridell *et al*. 1998).

In *cpe*-positive strains with *cpe* in the plasmid (plasmid-borne *cpe* strains), *cpe* is also closely associated with IS elements. Some plasmid-borne *cpe* strains possess sequences homologous to IS*1470* downstream of *cpe* (Miyamoto *et al*. 2002) (Fig. 1). These sequences reside in about the same position, but are oppositely oriented and defective relative to the IS*1470* sequences found in chromosomal *cpe*. The other plasmid-borne *cpe* strains contain an IS*1151* sequence downstream from *cpe* (Fig. 1). *cpe* plasmid with IS*1470*-like sequences has been shown *in vitro* to be transferable via conjugation to *cpe*-negative *C. perfringens* type A strains (Brynestad *et al*. 2001).

In addition, all currently investigated *cpe*-positive type A isolates contain IS*1469* about 1.2 kb upstream of *cpe,* and plasmid-borne *cpe* type A strains possess an ORF potentially encoding cytosine methylase (*dcm*) upstream of IS*1469* (Miyamoto *et al*. 2002) (Fig. 1). Similar *dcm* sequences have also been detected in several *cpe*-negative *C. perfringens* isolates carrying plasmids, but not in type A isolates carrying a chromosomal *cpe* (Miyamoto *et al*. 2002) (Fig. 1).

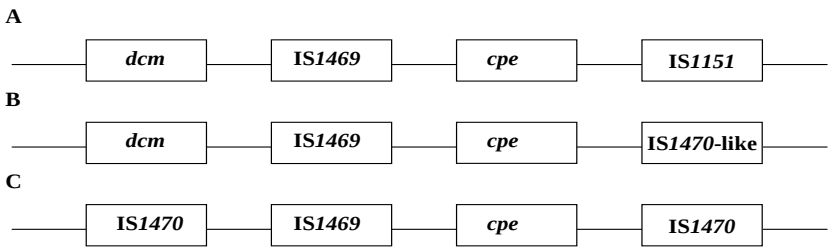

Figure 1. Location of different insertion sequence (IS) elements in relation to the location of *cpe* in *cpe*-positive *Clostridium perfringens* type A isolates. A, plasmid-borne *cpe* genotype IS*1151*-*cpe*; B, plasmid-borne *cpe* genotype IS*1470*-like-*cpe*; C, chromosomal genotype IS*1470*-*cpe* (Brynestad 1997, Miyamoto *et al*. 2002).

2.2.3 Sporulation and production of CPE

Spore formation is a trait of certain bacteria, including *Bacilli* and *Clostridia*, to survive under such unfavorable conditions imposed by heat, chemicals, radiation, or starvation (Stephenson and Lewis 2005). Spores are metabolically inactive and may survive for long periods, eventually resuming vegetative cell growth when appropriate conditions are present (Stephenson and Lewis 2005). Sporulation mechanisms have been extensively studied, but specific signals triggering *C. perfringens* sporulation are not well understood (Huang 2007).

On the genetic level, the initiation of sporulation is controlled by the sporulation transcription factor s*po0A* (Burbulys *et al*. 1991, Brown *et al*. 1994, Huang and Sarker 2006). Recently, it has been suggested that inorganic phosphate may serve as an important signal required for spore formation of *C. perfringens* (Philippe *et al*. 2006).

CPE biosynthesis is temporally associcated with sporulation (Duncan *et al*. 1972, Duncan 1973, Garcia-Alvarado *et al*. 1992, Czeczulin *et al*. 1996). CPE synthesis begins after the induction of sporulation and increases progressively for at least 6-8 hours (Smith and McDonel 1980, Melville *et al*. 1994). CPE is not secreted, but accumulates in the cytoplasm, and is localized occasionally in paracrystalline inclusion bodies (Duncan *et al*. 1973). CPE may represent up to 15-30% of the total protein present in the cell (Labbé 1981, Czeczulin *et al*. 1993, Melville *et al*. 1997). The toxin is released into the intestinal lumen when sporulation is completed and the mother cell lysed, releasing a mature spore (Duncan 1973).

The association between sporulation and CPE production has been evidenced also at the genetic level since inactivation of *spo0A* from a *cpe*-positive *C. perfringens* type A strain was shown to block not only sporulation but also CPE production (Huang *et al*. 2004).

2.2.4 Mechanism of action of CPE

Intestinal activity of CPE has been demonstrated in various animals (Duncan *et al*. 1968, Hauschild *et al*. 1970, Hauschild *et al*. 1971, Niilo and Dorward 1971, Bartlett *et al*. 1972, McDonel 1974, McDonel and Duncan 1975). Early studies showed that shortening of intestinal villi and desquamation of intestinal epithelium cause diarrheic symptoms (McDonel *et al*. 1978, Sherman *et al*. 1994). *In vitro* studies in the human ileum have revealed that CPE induces significant morphological damage and transport changes (Fernández-Miyakawa *et al*. 2005). Moreover, slight changes in the human colon have been seen after exposure to CPE (Fernández-Miyakawa *et al*. 2005). Intravenously administered CPE has been shown to be lethal for several animals (Niilo 1973, Siarakas *et al*. 1995).

On the cellular and molecular level, CPE inserts itself into the plasma membrane of enterocytes and forms a small complex of ~90 kDa with claudin-3 and claudin-4 receptors (Katahira *et al*. 1997a, Katahira *et al*. 1997b, Fujita *et al*. 2000, Smedley and McClane 2004). After forming the small complex with the receptor, CPE localizes to a larger, SDS-resistant complex (~155 kDa). This large complex initiates membrane permeability alterations, leading to calcium influx into the cell. Depending on the amount of CPE, cellular levels of calcium increase rapidly, and the cell dies via either the apoptotic or the oncotic pathway (Smedley and McClane 2004).

2.2.5 CPE-associated diseases

Food poisoning

C. perfringens type A is reported to be one of the most common causes of food poisonings throughout the industrialized world (Mead *et al*. 1999, Adak *et al*. 2002, Brynestad and Granum 2002, Lukinmaa *et al*. 2002, Hatakka and Pakkala 2003). The symptoms are caused

by CPE, and although other types of *C. perfringens* may also produce CPE, most (if not all) of these food poisoning cases are caused by *C. perfringens* type A strains (McClane 2001).

C. perfringens type A food poisoning outbreaks are usually reported in institutionalized settings and involve large numbers of victims (Lund *et al*. 2000). Temperature abuse of the food is considered the major contributing factor to this food poisoning, with the most common vehicle being meat or poultry. Optimal conditions for food poisonings arise when contaminated food is held or served at a temperature range of 10-54°C, allowing growth of the organism. When large numbers of vegetative cells are subsequently ingested, they sporulate and release CPE into the intestinal lumen (McClane 2001). As a consequence of this, severe abdominal cramps and diarrhea occur within 8-12 hours, usually with a spontaneous recovery within 24 hours. Fatalities are rare but possible in elderly or debilitated persons (Smith 1998, Bos *et al*. 2005).

Recommended laboratory criteria used in association with clinical presentation and epidemiological evidence to implicate *C. perfringens* in foodborne disease are as follows: high numbers of viable cells ($>10^5$/g) in suspect foods, presence of elevated fecal spore counts ($>10^6$/g), presence of the same serotype in all individuals in an outbreak, or demonstration of the same serotype of *C. perfringens* in both contaminated food and feces (Hauschild 1975, NCFA 1995, Lund *et al*. 2000). Notably, these widely used laboratory criteria do not involve the detection of CPE production or the presence of *cpe* gene in foods or feces, or in the suspected *C. perfringens* isolate. This is definitely a weakness since not all *C. perfringens* isolates present in food or feces produce CPE and are thus capable of causing food poisonings. Nor do traditionally used laboratory criteria involve molecular typing methods to determine the clonality of *C. perfringens* isolates related to the food poisoning outbreak.

Antibiotic-associated diarrhea and sporadic diarrhea

CPE-producing *C. perfringens* type A has also been implicated as a cause of up to 15% of antibiotic-associated diarrheas (AADs) and sporadic diarrheas (SDs) in humans (Borriello *et al*. 1984, Borriello *et al*. 1985, Jackson *et al*. 1986b, Brett *et al*. 1992, Mpamugo *et al*. 1995, Abrahao *et al*. 2001, Modi and Wilcox 2001, Asha and Wilcox 2002, Carney *et al*. 2002, Heimesaat *et al*. 2005, Joshy *et al*. 2006a, Joshy *et al*. 2006b). AADs develop after exposure to such antibiotic as penicillin, cephalosporins, trimethoprim, or cotrimoxazole (Borriello

1985, Borriello *et al*. 1985, Jackson *et al*. 1986b, Brett *et al*. 1992, Borriello 1995, Mpamugo *et al*. 1995, Modi and Wilcox 2001, Asha and Wilcox 2002, Ackermann *et al*. 2005). The symptoms involve watery and/or bloody diarrhea that can last for several weeks or months (Modi and Wilcox 2001). Relapses are also common (Borriello *et al*. 1984, Modi and Wilcox 2001). Risk factors are use of a nasogastric tube or proton pump inhibitors and older age of the patient (Asha *et al*. 2006). *C. perfringens* type A SD is suspected when a patient develops the disease without prior exposure to antibiotics (Wada *et al*. 1996).

The pathogenesis of AAD and SD caused by *cpe*-positive *C. perfringens* type A is not fully understood. In contrast to the massive numbers of *cpe*-positive cells ingested during *C. perfringens* type A food poisoning, AAD or SD is thought to be caused by a small inoculum of *cpe*-positive cells (Smedley *et al*. 2004). *In vivo* conjugative transfer of the *cpe* plasmid to normal intestinal flora isolates of *C. perfringens* has been suggested to be important in establishing *C. perfringens* type A AAD or SD. Small numbers of *cpe*-positive cells could transfer the *cpe* plasmid to the formerly *cpe*-negative *C. perfringens* strains that are already adapted for intestinal colonization and growth. This *in vivo* conversion would thus hasten disease onset and may even be required to reach the *in vivo* levels of *cpe*-positive *C. perfringens* cells necessary for initiating enteric disease (Brynestad *et al*. 2001). Conjugative transfer of *cpe* plasmid has been evidenced *in vitro*, but not yet *in vivo* (Brynestad *et al*. 2001). It is not known, whether these plasmid *cpe*-carrying *C. perfringens* type A strains are normally present in the GI tract or whether they are acquired just prior to disease onset. *Clostridium perfringens* beta2 toxin possibly acts as an accessory toxin in AAD and SD caused by *cpe*-positive *C. perfringens* type A (Fisher 2006).

Sudden infant death syndrome

Sudden infant death syndrome (SIDS) continues to account for large numbers of unexpected infant deaths in many countries, being considered a complex, multifactorial disorder affected by genetic, developmental, and environmental factors (Hunt and Hauck 2006). Bacterial toxins, such as *Clostridium perfringens* enterotoxin (CPE), have been proposed to play a role in SIDS (Murrell *et al*. 1987, Lindsay *et al*. 1993, Das 2002, Blackwell *et al*. 2005, Kamaras *et al*. 2001a, Kamaras *et al*. 2001b). Intestinal production and systemic absorption of CPE have been suggested to result in rapid death in SIDS, whereas in enteric diseases CPE acts

locally by damaging the intestinal epithelium while remaining unabsorbed from the lumen (Siarakas *et al*. 1995, Siarakas *et al*. 1999).

Veterinary diarrheas

CPE-producing *C. perfringens* type A has also been linked to diarrheic conditions in several animal species, including dogs, pigs, horses, and cows (Wierup 1977, Prescott *et al*. 1978, Wierup and DiPietro 1981, Carman and Lewis 1983, Nabuurs *et al*. 1983, Collins *et al*. 1989, Estrada-Correa *et al*. 1989, Kruth *et al*. 1989, Kanoe *et al*. 1990, Songer 1996, Marks and Kather 2003a, Marks and Kather 2003b, Cave *et al*. 2002). Moreover, dogs have been demonstrated to possess hospital-acquired diarrhea due to *cpe*-positive *C. perfringens* (Kruth *et al*. 1989).

2.2.6 Therapeutic use of CPE

Claudin-3 and claudin-4 receptors are highly expressed in several cancer types (Long *et al*. 2001, Michl *et al*. 2001, Kominsky *et al*. 2004, Nichols *et al*. 2004, Santin *et al*. 2007). Therefore, the ability of CPE to rapidly and specifically lyse cells expressing claudin-3 and claudin-4 may be useful in the treatment of these cancers types (Long *et al*. 2001, Michl *et al*. 2001, Kominsky *et al*. 2004, Nichols *et al*. 2004, Santin *et al*. 2007). In addition to type-specific therapy for tumors, CPE could aid in drug delivery by being a potent absorption enhancer (Ebihara *et al*. 2006, Kondoh *et al*. 2006).

2.3 Diagnostics of *cpe*-positive *Clostridium perfringens* type A

2.3.1 Culture methods and confirmation tests

C. perfringens is considered relatively easy to isolate. Its short generation time and relatively high optimal growth temperature (43-45ºC) facilitate isolation among most competing organisms (Smith 1975). The ability of *C. perfringens* to form heat-resistant spores also facilitates isolation since most of the competing organisms are killed by heating (Smith 1975). Moreover, the tolerance of *C. perfringens* for low concentrations of oxygen also offers an

advantage (Smith 1975, Labbé 2000). Conventional detection of *C. perfringens* is based on cultivation and isolation of typical colonies from media, following by confirmation of colonies with different tests to reveal phenotypic characteristics. Relative resistance to such compounds as sodium sulfite, sulfadiazine, polymyxin, neomycin, kanamycin, and cycloserine has enabled the development of several media for *C. perfringens* cultivation and isolation, including tryptose-sulfite-cycloserine (TSC) media with or without egg yolk, oleandomycin-polymyxin-sulfadiazine-perfringens (OPSP), and blood agar (BA) with neomycin (Harmon *et al*. 1971, Hauschild and Hilsheimer 1974a, Hauschild and Hilsheimer 1974b, Hauschild *et al*. 1974, Labbé 2000). Observation of nitrate reduction, gelatine liquefaction, lactose fermentation, or lack of motility in suspected *C. perfringens* isolates is usually sufficient to distinguish *C. perfringens* from other organisms (Cato *et al*. 1986). Identification of *C. perfringens* may be also accomplished using commercial biochemical test kits such as API (Labbé 2000). Several international method standards are available for confirmation of *C. perfringens* in different samples, including methods published by the Association of Official Analytical Chemists (AOAC), the International Standardization Organization (ISO), and the Nordic Committee on Food Analysis (NCFA).

2.3.2 Sporulation of *Clostridium perfringens* and detection of CPE

CPE is synthesized during sporulation, and thus, sporulation *in vitro* is essential to measure the production of CPE of an isolate. Several sporulation media have been devised for sporulation of *C. perfringens* (Ellner 1956, Kim *et al*. 1967, Duncan and Strong 1968, Taniguti 1968, Nishida *et al*. 1969, Sacks and Thompson 1978, Tortora 1984, Tortora and Costa 1985, Harmon and Kautter 1986, Meyer and Tholozan 1999). However, reproducible sporulation of *C. perfringens* is challenging, depending on both the strain and the medium used (Labbé 1989). Replacement of starch with raffinose has been found to improve the sporulation in some strains (Labbé and Rey 1979). Also addition of caffeine, theophylline, or theobromine can increase spore yields in certain strains (Labbé and Nolan 1981, Sacks and Thompson 1975, Sacks and Thompson 1977, Sacks and Thompson 1978, Lane and Philadelphia 1993). Historically, cooked meat has been the predominant medium applied (Labbé 1989), and due to the spore-producing characteristics of this medium, it is often used for preparing stock cultures of *C. perfringens*. Today, modified Duncan Strong medium is perhaps most commonly used in sporulating *C. perfringens* (Lund *et al*. 2000). The medium

contains protease peptone, yeast extract, sodium thioglycollate, sodium hydrogenphosphate (Na_2HPO_4), and raffinose.

The classic way to detect CPE is by observing morphological changes in rabbit ileum in the rabbit ileal loop test (Duncan *et al*. 1968). Guinea-pig skin test, mouse test, and Vero cell assay are other biological methods for detecting CPE (Mahony *et al*. 1989, McDonel and McClane 1981). Serological methods are based on using monoclonal or polyclonal antibodies. The first serological methods involved microslide single- or double-gel diffusion (Genigeorgis *et al*. 1973, Tortora and Costa 1985), counterimmunoelectrophoresis (Naik and Duncan 1977) and electroimmunodiffusion (Duncan and Somers 1972). In these assays, detection of CPE is based on observing a precipitation line between CPE and the corresponding antiserum. Reverse passive hemagglutination has also been used (Uemura *et al*. 1973). Flow cytometry has been used as an aid to detect cytotoxic activity of CPE (Hu *et al*. 1995, Kusunoki *et al*. 1998). A widely used technique, with a number of assays available, is the enzyme-linked immunosorbent assay (ELISA) (Olsvik *et al*. 1982, McClane and Strouse 1984, Notermans *et al*. 1984, Bartholomew *et al*. 1985, Jackson *et al*. 1985, Jackson *et al*. 1986a, Wimsatt *et al*. 1986, Berry *et al*. 1988, Mehta *et al*. 1989, Piyankarage *et al*. 1999). Methods based on immunomagnetic separation (IMS) and ELISA as well as chemiluminescent enzyme immunoassay (EIA) have also been reported (Cudjoe *et al*. 1991, Baez *et al*. 1996). A kit based on reverse passive latex agglutination (RPLA) is extremely popular since it provides a straightforward method for detection of CPE from clinical samples (Berry and Stringer 1985, Berry *et al*. 1986, Berry *et al*. 1988). Western immunoblotting offers a highly specific serologic assay for CPE detection, identifying the 35-kDa CPE using antibodies prepared against purified CPE (Kokai-Kun *et al*. 1994). However, this method is laborious and is thus primarily used for research purposes.

In general, serological methods have largely replaced biological methods in detecting CPE, as they are usually considered more sensitive in detecting low numbers of CPE (Petit *et al*. 1999, Robinson *et al*. 2000). Some of these methods achieve a sensitivity within the nanogram range, whereas biological methods are usually within the microgram range.

2.3.3 Detection of *cpe*

The early methods for detecting *cpe* were based on DNA hybridization using a probe specific for *cpe* or parts of the gene (Van Damme-Jongsten et al. 1990, Kokai-Kun et al. 1994, Daube et al. 1996, Tschirdewahn et al. 1991). Several polymerase chain reaction (PCR) methods have since been established (Table 4). These methods amplify *cpe* from total bacterial DNA or bacterial cell lysate or directly from clinical or food samples. Other methods, such as DNA microarray, have also been reported in detecting *cpe* and other toxin genes (Al-Khaldi *et al*. 2004a, Al-Khaldi *et al*. 2004b). Colony hybridization has been used to detect *cpe* in raw beef (Baez and Juneja 1995).

Table 4. PCR methods to detect *cpe* gene alone or in combination with other toxin genes.

Gene(s) detected	**Specimen(s)**	**Application**	**Reference**
cpe	Bacterial cell lysate, DNA	Single PCR	(Kokai-Kun *et al*. 1994, Ridell *et al*. 1998)
	Feces	Single PCR	(Saito *et al*. 1992)
	Meat, feces	Nested PCR	(Miwa *et al*. 1996)
	Intestinal contents	MPN-PCR	(Miwa *et al*. 1997)
	Various foods	Single PCR	(Kim *et al*. 2000)
	Meat	PCR-ELISA	(Baez *et al*. 1996)
cpe, *cpa*	Bacterial cell lysate, DNA	Duplex PCR	(Tansuphasiri 2001, Augustynowicz *et al*. 2002)
	Food, feces	Duplex PCR	(Fach and Popoff 1997)
	Feces, intestinal contents	Duplex PCR	(Kanakaraj *et al*. 1998)
	Feces	Duplex PCR	(Tansuphasiri *et al*. 2002)
	Water, food, stool samples	Real-time FRET-PCR	(Cruz *et al*. 2006)
cpe, cpa, cpb, etx, ia	Bacterial cell lysate, DNA	Multiplex PCR	(Daube *et al*. 1994, Songer and Meer 1996, Meer and Songer 1997, Kadra *et al*. 1999, Kalender *et al*. 2005)
cpe, cpa, cpb, etx, ia, cpb2	Bacterial cell lysate, DNA	Multiplex PCR	(Herholz *et al*. 1999, Garmory *et al*. 2000, Gkiourtzidis *et al*. 2001, Baums *et al*. 2004)

2.3.4 Molecular typing of *Clostridium perfringens*

Typing is the assessment of the relatedness of a group of bacterial isolates, aimed at determining whether or not they originate from a common source (Tenover *et al*. 1995). Several typing systems, based on either phenotypic or genotypic characteristics of the organism, have been applied in typing *C. perfringens* (Table 5). Traditionally, serotyping has been used as an epidemiological tool in the investigation of *C. perfringens* food poisoning outbreaks, (Hauschild 1975, Lund *et al*. 2000). Currently, the most commonly used typing method in epidemiological studies of *C. perfringens* is perhaps pulsed-field gel electrophoresis (PFGE) (Table 5), being highly reproducible and discriminatory (Schwartz and Cantor 1984, Tenover *et al*. 1997). However, in some cases, PFGE has been unable to characterize isolates of *C. perfringens* or other *Clostridia* due to degradation of DNA by DNAses (Kato *et al*. 1994, Wada *et al*. 1996, Hielm *et al*. 1998, Schalch *et al*. 2003).

Recently, PCR genotyping assays have been also developed to detect different IS elements attached to *cpe*, and thus, to establish whether *cpe*-positive *C. perfringens* type A isolates carry chromosomal or plasmid-borne *cpe* (Wen *et al*. 2003, Miyamoto *et al*. 2004).

Table 5. Typing methods used for determining the relatedness of *Clostridium perfringens* isolates obtained from humans, animals, and foods.

Typing method	**Reference(s)**
Bacteriocin typing	(Mahony 1974, Mahony and Swantee 1978, Satija and Narayan 1980, Watson *et al*. 1982, Watson 1985, Mahony *et al*. 1992, Higa *et al*. 1991, Schalch *et al*. 1998)
Serotyping	(Hughes et al. 1976, Stringer *et al*. 1980, Thomas and Noah 1977, Chakrabarty and Narayan 1979, Smart *et al*. 1979, Gross *et al*. 1989)
Phage typing	(Yan 1989)
Plasmid profile typing	(Mahony *et al*. 1987, Jones *et al*. 1989, Mahony *et al*. 1992, Eisgruber *et al*. 1995, Eisgruber *et al*. 1996, Schalch *et al*. 1998, Schalch *et al*. 1999)
Ribotyping	(Forsblom *et al*. 1995, Schalch *et al*. 1997, Schalch *et al*. 1998, Kilic *et al*. 2002, Manteca *et al*. 2002, Schalch *et al*. 2003)
Pulsed-field gel electrophoresis typing (PFGE)	(Wada *et al*. 1996, Collie *et al*. 1998, Ridell *et al*. 1998, Maslanka *et al*. 1999, Lukinmaa *et al*. 2002, Bangsberg *et al*. 2002, Pituch *et al*. 2002, Engstrom *et al*. 2003, Lin and Labbé 2003, Nauerby *et al*. 2003, Nakamura *et al*. 2003, Schalch *et al*. 2003, Fukao *et al*. 2004, Sobel *et al*. 2005, Gholamiandekhordi *et al*. 2006, Johansson *et al*. 2006)
Multilocus sequence typing (MLST)	(Jost *et al*. 2006)
Multilocus enzyme typing	(Pons *et al*. 1994)
Multilocus variable-number tandem repeat analysis (MLVNTR)	(Sawires and Songer 2005)
Randomly amplified polymorphic DNA (RAPD)	(Leflon-Guibout *et al.* 1997)
Repetitive element PCR (rep-PCR)	(Siragusa *et al*. 2006)
Amplified fragment length polymorphism (AFLP)	(McLauchlin *et al*. 2000, McLauchlin *et al*. 2002, Settings 2006)

2.4 Epidemiology of *cpe*-positive *Clostridium perfringens* type A

Since meat and poultry products are the most common vehicles of *C. perfringens* type A food poisoning, contamination of the meat by the intestinal contents of slaughtered animals might

serve an important source of this pathogen to the food supply (Labbé 2000, Heredia and Labbé 2001). However, because *C. perfringens* spores are ubiquitously distributed, the contamination might also occur elsewhere in the food chain (McClane 2001). Despite wide dispersion of *C. perfringens*, CPE-producing isolates are only occasionally isolated. These have been estimated to represent less than 5% of global *C. perfringens* isolates (Smedley *et al*. 2004) (Table 6). Thus, the reservoirs for *cpe*-positive *C. perfringens* type A are not currently understood, and no clear picture has emerged regarding the transmission routes of *cpe*-positive *C. perfringens* type A isolates to the food chain.

Recent findings of the variable loci (plasmid vs. chromosome) and different genetic arrangements adjacent to *cpe* have shown that *cpe*-positive isolates form different subpopulations. These different subpopulations have been found to be responsible for different CPE-associated diseases; chromosomal *cpe*-bearing strains are involved in food poisonings, whereas strains carrying *cpe* in the plasmid are typical in antibiotic-associated or sporadic diarrhea cases as well as in animal diarrheas (Cornillot *et al*. 1995, Katayama *et al*. 1996, Collie and McClane 1998, Sparks *et al*. 2001). Reasons for the strong disease-genotype relationship have been elucidated. *C. perfringens* strains carrying chromosomal *cpe* have been found to be more resistant to heating, osmotic stress, and low temperatures than plasmid-borne *cpe*-carrying strains (Sarker *et al*. 2000, Li and McClane 2006a, Li and McClane 2006b), and thus, their presence in retail foods (Wen and McClane 2004) and their predominance in food poisonings (Smedley *et al*. 2004) are understandable. Chromosomal *cpe* strains may gain access to foods more easily and take advantage of temperature abuse more effectively than strains with plasmid-borne *cpe*. Although the disease-genotype relationship has been described in several studies, no research has been conducted to elucidate the reservoirs for different genotypes.

Table 6. Prevalence of *cpe*-positive *Clostridium perfringens* in healthy animals, humans, foods, and the environment.

Sample		**Number of samples studied**	**Number of *C. perfringens* isolates studied**	**Prevalence of *cpe*-positive *C. perfringens* in samples (%)**	**Prevalence of total *C. perfringens* in samples (%)**	**Detection method[a]**	**Country**	**Reference**
Feces or intestines	Calves	21[b]	NK[c]	10	NK	ELISA	France	(de Rycke *et al*. 1986)
	Cats	8	NK	0	50	RPLA, PCR	Germany	(Werdeling *et al*. 1991)
		22[b]	171	1	NK	CH	Belgium	(Daube *et al*. 1996)
	Cattle	51[b]	35	0	49	RPLA	Japan	(Saito 1990)
		50	0	26	76	MPN- nested PCR	Japan	(Miwa *et al*. 1997)
		10	0	10	NK	Nested PCR	Japan	(Miwa *et al*. 1996)
		50[b]	NK	22	36	Dot blot	Switzerland	(Tschirdewahn *et al*. 1991)
		390[b]	1443	0.4[d]	NK	CH	Belgium	(Daube *et al*. 1996)
	Dogs	39	NK	2[b]	54	RPLA, PCR	Germany	(Werdeling *et al*. 1991)
		106[b]	134	2	98	RPLA	Japan	(Saito 1990)
		35	200	3[b]	NK	CH	Belgium	(Daube *et al*. 1996)
		16	77	13	NK	CH	Belgium	(Daube *et al*. 1996)
	Fowl	76[b]	11	0	14	RPLA	Japan	(Saito 1990)
		50	0	40	80	MPN- nested PCR	Japan	(Miwa *et al*. 1997)
		59[b]	NK	10	80	Dot blot	Switzerland	(Tschirdewahn *et al*. 1991)
		40	42	0[b]	NK	CH	Belgium	(Daube *et al*. 1996)
	Goats	20	55	4[b]	NK	CH	Belgium	(Daube *et al*. 1996)

	Horses	50[b]	NK	14	24	Dot blot	Switzerland	(Tschirdewahn *et al*. 1991)
	Humans	80[b]	55	6	57	RPLA	Japan	(Saito 1990)
		35	NK	31	NK	RPHA	Japan	(Uemura 1978)
		46	224	6[b]	NK	CH	Belgium	(Daube *et al*. 1996)
		200	225	7	63	Dot blot	Mexico	(Vela *et al*. 1999)
	Reindeer	166	98	2	59	PCR	Norway	(Aschfalk *et al*. 2002)
	Sheep	194	61	0	31	PCR	Turkey	(Kalender *et al*. 2005)
		63	219	2[b]	NK	CH	Belgium	(Daube *et al*. 1996)
	Swine	358	62	0	25	PCR	USA	(Kanakaraj *et al*. 1998)
		131[b]	88	0	55	RPLA	Japan	(Saito 1990)
		50	0	22	44	MPN- nested PCR	Japan	(Miwa *et al*. 1997)
		10	0	0	NK	nested PCR	Japan	(Miwa *et al*. 1996)
		50[b]	NK	0	2	Dot blot	Switzerland	(Tschirdewahn *et al*. 1991)
		48[b]	63	2[b]	NK	CH	Belgium	(Daube *et al*. 1996)
Meat (raw)	Beef	50	0	2	16	MPN-PCR	Japan	(Miwa *et al*. 1998)
		10	0	0	NK	Nested PCR	Japan	(Miwa *et al*. 1996)
	Pork	50	0	0	10	MPN-PCR	Japan	(Miwa *et al*. 1998)
		10	0	0	NK	PCR	Japan	(Miwa *et al*. 1996)
	Chicken	50	0	12	84	MPN-PCR	Japan	(Miwa *et al*. 1998)
Meat (processed)	Sausage	75	NK	8[b]	NK	RPLA	Costa Rica	(Morera *et al*. 1999)
		315	83	2.5	26	RPLA, PCR	Argentina	(Virginia *et al*. 2002)
	Hamburger	100	19	0	19	RPLA, PCR	Argentina	(Virginia *et al*. 2002)
	Minced meat	100	24	1	24	RPLA, PCR	Argentina	(Virginia *et al*. 2002)
Retail food	Animal origin	900	NK	1.4	31	PCR	USA	(Wen and McClane 2004)
	Animal and nonanimal origin	131	NK	0	30	PCR	USA	(Lin and Labbé 2003)

Spices	Various different origins	115	14	3	12	RPLA, PCR	Argentina	(Aguilera *et al*. 2005)
		380	188	4[b]	NK	Dot blot	Mexico	(Rodriguez-Romo *et al*. 1998)
	Curry roux	60	NK	0	12	RPLA	Japan	(Fujisawa *et al*. 2001)
Swine feed		60	0	0	48	PCR	USA	(Kanakaraj *et al*. 1998)
Miscellaneous	Animals and humans, animal and human food, unknown origin[a]	NK	616[a]	8[b]	NK	PCR	USA and Canada	(Songer and Meer 1996)
	Animals and humans, animal food, unknown origin	NK	454	4	NK	PCR	USA	(Kokai-Kun *et al*. 1994)

[a] Detection methods: ELISA, enzyme-linked immunosorbent assay; RPLA, reversed passive latex agglutination; PCR, polymerase chain reaction; CH, colony hybridization; MPN-nested PCR, nested PCR combined with the most probable number technique; RPHA, reversed passive hemagglutination.

[b] Some of the studied samples associated with diseases.

[c] NK, not known.

[d] Prevalence of *cpe*-positive isolates among studied *C. perfringens* isolates.

3. AIMS OF THE STUDY

Studies I-V aimed to develop diagnostics and to investigate the molecular epidemiology of *cpe*-positive *C. perfringens* type A. Specific aims were as follows:

1. To develop a method for detection, enumeration, and isolation of *cpe*-positive *C. perfringens* spores from feces (I).

2. To establish a multiplex polymerase chain reaction assay for toxinotyping *C. perfringens* isolates by modifying a previously published protocol (II).

3. To develop an amplified fragment length polymorphism protocol for typing *C. perfringens* isolates and for distinguishing *C. perfringens* isolates from isolates of other *Clostridia* species (III).

4. To examine the molecular epidemiology of *cpe*-positive *C. perfringens* type A by elucidating the role of healthy food-handlers as a reservoir and determining the involvement of different genotypes of *cpe*-positive *C. perfringens* type A in food poisonings (IV and V).

4. MATERIALS AND METHODS

4.1 Bacterial strains and culturing (I-V)

A total of 11 *cpe*-positive and 19 *cpe*-negative *C. perfringens* strains, 38 strains of other clostridia, and nine strains of other bacterial species were included in the development of the method for enumeration and isolation of *cpe*-positive *C. perfringens* (I). In Study II, a total of 10 different *C. perfringens* strains, representing different toxin types, and 15 strains of other bacterial species were analyzed, and 118 *C. perfringens* isolates from broiler chickens were evaluated (II, Table 1). In Study III, 129 clostridial strains, 37 of which were *C. perfringens* strains, were used to establish the AFLP protocol (III, Table I). In Study V, a collection of 53 *C. perfringens* isolates, 26 from patients and 27 from various foods associated with 11 Finnish and 13 German food poisoning outbreaks during 1984-2007, were evaluated (V, Table 1). In Studies IV and V, *C. perfringens* strains NCTC 8239 (type A, *cpe*-positive), ATCC 3626 (type B), CCUG 2036 (type C), CCUG 2037 (type D), and CCUG 44727 (type E) were used as reference strains. *C. perfringens* strains were cultivated on blood agar plates and incubated at 37ºC for 20-24 h under anaerobic conditions. Other strains were incubated at their optimal temperatures and atmospheres.

4.2 Sampling (I and IV)

A total of seven fecal specimens (4 from females and 3 from males) were collected from healthy individuals for the development of the enumeration and isolation method (I). The ages of these persons varied from 17 to 57 years, and none reported any GI symptoms at the time of sampling. In Study IV, fecal specimens of 136 food-handlers (102 females and 34 males) were investigated. The specimens were collected in Southern Finland during summer 2003. The ages of these persons varied from 15 to 65 years, and none reported any GI symptoms at the time of sampling. All specimens were kept at -70ºC prior to use.

4.3 DNA extraction (I-V)

In Study I, total DNA from clostridial strains was isolated as described by Hyytiä *et al*. (1999), and from *Listeria* strains by using the protocol of Keto-Timonen *et al*. (2005) In Studies II, IV, and V, *C. perfringens* DNA was isolated by using Advamax beads (Edge Biosystems, Gaithersburg, MD, USA) according to the manufacturer's instructions. In Study III, the protocol described by Hyytiä et al. (1999) was used, with slight modifications. Strains were grown in a tryptose-peptone-glucose-yeast medium (Difco Laboratories, Detroit, MI, USA) under anaerobic conditions at the optimal growth temperature for each strain for 14-16 h. The cells were resuspended in 400 µl of TE (10 mM Tris-HCl, 1 mM EDTA) and incubated with 7.9 mg/ml lysozyme (Sigma, St. Louis, MO, USA), 159 IU/ml mutanolysin (Sigma), and 476 µg/ml RNase (Sigma) at 37°C with gentle shaking for 15 min (*C. botulinum* group I), 2 h (*C. botulinum* group II), or 1 h (other *Clostridium* species). Lysis was completed by adding 52 µg/ml proteinase K (Finnzymes, Espoo, Finland), 0.23 M NaCl, 9.1 mM EDTA, and 0.8% (vol/vol) sodium dodecyl sulfate. After thorough mixing, the mixture was incubated at 60°C for 1 h with gentle shaking. Phenol-chloroform-isoamyl alcohol (25:24:1 [vol/vol]) and chloroform-2-pentanol (24:1 [vol/vol]) extractions were performed, and the DNA was precipitated with ethanol (95% [vol/vol]), rinsed with 70% ethanol, and resuspended in 100 µl of sterile, distilled, deionized water; DNA was stored at –70°C. DNA concentrations were determined using a BioPhotometer (Eppendorf, Hamburg, Germany).

4.4 Enumeration and isolation of *cpe*-positive *Clostridium perfringens* (I)

4.4.1 *cpe*-specific probe preparation and sensitivity and specificity testing (I)

A 425-bp, DIG-labeled DNA probe, specific for *cpe*, was prepared by PCR (Miwa *et al*. 1996) and labelling kits (High Pure PCR Product Purification Kit, Roche, Mannheim, Germany) (DIG High Prime DNA Labeling and Detection Starter Kit I, Roche). The sensitivity of the probe was tested as recommended by the manufacturer. The specificity of the probe was tested by hybridizing bacterial DNA or pure bacterial cultures. Bacterial DNA was spotted on nylon membranes (Roche) and fixed onto membranes using UV crosslinker

(Spectroline, Spectronics Corporation, Westbury, NY, USA). Subsequently, the membranes were prehybridized at 41°C for 1 h (DIG Easy Hyb, Roche) and hybridized (DIG Easy Hyb with 25 ng/ml of denatured probe) overnight at 41°C. Membranes were then washed (2 × SSC with 0.1% SDS) and the hybrids were detected with a chromogenic assay using the protocol provided by the DIG Application Manual (Roche).

When testing pure bacterial cultures, strains were cultivated on hydrophobic grid membrane filters (HGMFs) (Iso-Grid, Neogen, Baltimore, MD, USA), incubated on tryptose-sulfite-cycloserine (TSC) plates (Shahidi Ferguson perfringens agar, Difco Laboratories, Detroit, MI, USA, supplemented with 1% D-cycloserine, Sigma-Aldrich, St. Louis, MO, USA) under anaerobic conditions at 37°C for 24 h. Bacterial growth on the HGMF-TSC plates was replicated on nylon membranes. The membranes were denatured (0.5 M NaOH, 1.5 M NaCl), neutralized (1.0 M Tris-HCl, 1.5 M NaCl, pH 7.4), and air-dried. Exposed DNA was subsequently fixed to the membranes as described above. Cell debris was removed using proteinase K (Finnzymes, Espoo, Finland) and tightly pressing blotting paper onto the membranes. The membranes were thereafter prehybridized and hybridized, washed, and stained as described above.

4.4.2 Enumeration and isolation of *cpe*-positive *Clostridium perfringens* from fecal samples using the hydrophobic grid membrane filter- colony hybridization method (I)

A fecal sample (0.1 g) was diluted tenfold in 0.1% peptone water and heated at 75°C for 20 min and filtered using 10 HGMFs in a membrane filtration system (Iso-Grid, Neogen). HGMFs were placed on TSC plates and incubated for 36-42 h at 37°C. Bacterial growth on HGMF-TSC agar plates was replicated on nylon membranes, and these replicas were hybridized with the DIG-labeled DNA probe as described above with pure bacterial cultures. Positive hybridization signals on nylon membranes were enumerated, considered as *cpe*-positive colonies on the original HGMF-TSC plate. Nested PCR combined with the MPN technique (MPN-PCR) (Oblinger and Koburger 1984) (Miwa *et al*. 1996) was used as a reference to the quantification obtained by the HGMF-CH method. *cpe*-positive colonies on original HGMF-TSC plates were localized by using the nylon membranes. Several probe-positive colonies were streaked onto blood agar and incubated at 37°C for 24 h. Isolates were

further analyzed for the presence of the *cpe* gene by PCR (Miwa *et al*. 1996). In Study IV, the HGMF-CH method was used for enumeration and isolation of *cpe*-positive *C. perfringens* from the samples with a positive PCR result for the presence of *cpe*.

4.5 Multiplex polymerase chain reaction assay (II)

In Study II, the multiplex PCR assay was established by modifying a previously published protocol (Meer and Songer 1997). Modification was necessary because when using CPA primers of the published protocol, no amplification products were revealed despite several optimization attempts and testing with wild and reference *C. perfringens* strains. The novel assay was thus established by designing primers for *cpa* by using Primer3 program (http://www-genome.wi.mit.edu/genome_software/other/primer3.html) (II, Table 2). The target sequence was chosen from published DNA sequences of the *cpa* gene (Tsutsui *et al.* 1995, Genbank accession number L43547) (http://www.ncbi.nlm.nih.gov/Genbank/index.html). The size of the amplified product was adjusted to allow it to be incorporated into the previously published multiplex protocol (Meer and Songer 1997). A sequence homology search was performed by using BLAST (http://www.ncbi.nlm.nih.gov/blast) to confirm uniqueness of the sequence. The novel assay was tested by investigating 118 *C. perfringens* isolates from Finnish broiler chickens. The assay was also used in subsequent studies for toxinotyping and determining the presence of *cpe* in *C. perfringens* isolates (IV and V).

4.6 Amplified fragment length polymorphism method (III)

The previously used AFLP protocol for *C. botulinum* strains (Keto-Timonen *et al*. 2005) was applied with some modifications. Total DNA was digested by using a total of 15 U of *Hind*III (New England Biolabs) and 15 U of HpyCH4IV (New England Biolabs) in 1 × One-Phor-All buffer plus (Amersham Biosciences, Buckinghamshire, UK), 5 mM dithiothreitol, and 0.1 mg/ml bovine serum albumin. Restriction site-specific adapters for *Hind*III (0.04 μM; Oligomer, Helsinki, Finland) and HpyCH4IV (0.4 μM; Oligomer) were ligated with 1.1 U T4

ligase (New England Biolabs) in 1 × One-Phor-All buffer plus (Amersham Biosciences), 5 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, and 200 μM ATP.

After digestion and ligation, DNA samples were subjected to preselective PCR using 25 nM Hind-0 and 125 nM Hpy-0 primers (Oligomer) (sequences described in Table 2, Study III) in a 20-μl reaction mixture also containing 4 μl of diluted template DNA and 15 μl of amplification core mix (Applied Biosystems, Foster City, CA, USA). The protocol was as follows: 72ºC for 2 min; 20 cycles of 94ºC for 20s, 56ºC for 2 min, and 72ºC for 2 min.

Selective PCR was subsequently done in 10-μl reaction mixtures containing 50 nM 6-carboxyfluorescein (FAM)-labeled Hind-C primer (Oligomer), 250 nM Hpy-A primer (Oligomer), and 7.5 μl of amplification core mix (Applied Biosystems). The following protocol was used: 94ºC for 2 min, 1 cycle at 94ºC for 20 s, 66ºC for 30 s, 72ºC for 2 min, following by lowering of the temperature by 1ºC each cycle to 56ºC (10 cycles), then 19 cycles at annealing temperature (56ºC) and a final extension at 60ºC for 30 min. Peltier thermal cycler (MJ Research, Inc., Waltham, MA, USA) was used in all PCR reactions.

Internal size standard (0.5 μl) (GS-500 LIZ; Applied Biosystems) and Hi-Di formamide (11.5 μl) (Applied Biosystems) were mixed with selective amplification products (1 μl) and denatured at 95ºC for 2 min. The fragments were electrophoresed on POP-4 polymer (Applied Biosystems) on an ABI PRISM 310 Genetic analyzer (Applied Biosystems)in 1 × genetic analyzer buffer with EDTA (Applied Biosystems). The electrophoresis was done with 15 kV at 60ºC for 28 min. Data was preprocessed with Genescan 3.7 fragment analysis software (Applied Biosystems). AFLP patterns were analyzed using BioNumerics software 4.5 (Applied Maths, Kortrijk, Belgium). Similarity between patterns was calculated with Pearson product-moment correlation coefficient. Clustering and dendrogram construction were performed by using the unweighted pair-group method with arithmetic averages.

4.7 Detection of *cpe* in enriched samples (IV)

The presence of *cpe* was determined from enriched samples by using nested PCR (Miwa *et al*. 1996) (IV). Two different procedures with sample preparation were used; Samples were enriched both after heating the sample at 75ºC for 20 min and without the heating procedure.

4.8 Determination of the location of *cpe* and presence of different insertion sequence elements in *cpe*-positive *Clostridium perfringens* type A isolates (IV and V)

The locations of *cpe* and IS elements downstream from *cpe* (plasmid-borne *cpe* genotypes IS*1151*-*cpe* or IS*1470*-like-*cpe* and chromosomal *cpe* genotype IS*1470*-*cpe*) in *cpe*-positive *C. perfringens* type A isolates were determined by PCR using previously described primers (Daube *et al*. 1993, Brynestad 1997, Brynestad *et al*. 1997, Miyamoto *et al*. 2002, Miyamoto *et al*. 2004) and protocols (IV).

4.9 Pulsed-field gel electrophoresis analysis of *Clostridium perfringens* isolates (IV and V)

Restriction enzymes *Sma*I and *Apa*I (New England Biolabs, Beverly, MA, USA) were used for cleavage of *C. perfringens* DNA (IV and V). A previously used protocol was applied (Ridell *et al*. 1998), and PFGE profiles were analyzed either visually (V) or both visually and with a computer software program (Bionumerics, version 4.5; Applied Maths, Kortrijk, Belgium) (IV). The similarities between macrorestriction patterns (MRPs) were expressed by Dice coefficient correlation, and clustering by the unweighted pair-group method with arithmetic averages was used to construct a dendrogram.

4.10 Detection of CPE (IV and V)

cpe-positive *C. perfringens* isolates were sporulated in modified Duncan Strong medium (Sigma-Aldrich Chemie, Steinheim, Switzerland) (Miwa *et al*. 2002) (IV and V). Sporulation is essential prior to detection of CPE from a *cpe*-positive *C. perfringens* isolate, since CPE is formed in the cell during sporulation. Successful sporulation was confirmed by phase-contrast microscopy, and the culture was sonicated until more than 95% of the spores were free, determined by phase-contrast microscopy.

The cytotoxicity of the supernatant was tested using either Vero cell assay (Sandvig and Olsnes 1982) (IV) or reverse passive latex agglutination assay (PET-RPLA Seiken, Denka Seiken, Tokyo, Japan) (V). Vero cells were grown in a minimal essential medium (MEM; Gibco BRL, Paisley, UK) supplemented with 10% fetal calf serum. The strain was defined as producing CPE when the inhibition of protein synthesis of Vero cells was more than 20%. A reverse passive latex agglutination assay was conducted according to the instructions of the manufacturer.

5. RESULTS

5.1 Diagnostics of *cpe*-positive *Clostridium perfringens* type A (I-III)

The *cpe*-specific probe was found to be sensitive and specific when tested with *C. perfringens* DNA and cell lysate. The HGMF-CH method was as sensitive as MPN-PCR when tested with fecal samples from healthy individuals. With the aid of the HGMF-CH method, *cpe*-positive *C. perfringens* type A was successfully enumerated and isolated from fecal samples possessing positive hybridization signals. The number of *cpe*-positive *C. perfringens* type A in these samples varied from 60 to 1500 CFU/g. The detection limit of the method was dependent on the ratio of *cpe*-positive *C. perfringens* colonies to total *C. perfringens* growing on the HGMF-TSC plate. As each HGMF allows the growth of 1600 bacterial colonies, one *cpe*-positive *C. perfringens* colony could be detected among 1600 total *C. perfringens* colonies. Thus, when investigating 10 HGMFs, *cpe*-positive *C. perfringens* could be isolated when the ratio of *cpe*-positive *C. perfringens* to total *C. perfringens* was 6×10^{-5} or higher.

When establishing the multiplex PCR for toxinotyping and determining the presence of *cpe* from an isolate, all 10 *C. perfringens* reference strains yielded expected amplification products, and none of the other bacterial species; one *C. aerotolerans*, seven *C. botulinum*, one *C. chauvoei*, one *C. histolyticum*, one *C. sporogenes* and four *Staphylococcus aureus* isolates yielded PCR products. The PCR products were clearly visible and easily distinguishable from each other. The newly designed CPA primers were functional when used in both single PCR and multiplex PCR reaction with other primers. All 118 *C. perfringens* isolates possessed the *cpa* gene, but not *cpb, etx, iA,* or *cpe,* signifying that all isolates represented *C. perfringens* type A and were *cpe*-negative. The assay was further successfully used in Studies IV and V in determining the toxinotype (A-E) and carriage of *cpe* in *C. perfringens* isolates.

AFLP was found to be suitable for the identification of *C. perfringens* among other *Clostridium* species as well as for typing *C. perfringens* at the strain level. All *C. perfringens* strains were shown to be typeable by AFLP, and thus, the method overcame the occasional problem of extracellular DNase production encountered when typing clostridia isolates. AFLP

was highly reproducible, easy-to-perform, and relatively fast method, enabling high throughput of samples.

5.2 Role of the healthy food-handler as a reservoir for *cpe*-positive *Clostridium perfringens* type A (IV)

cpe was detected in the feces of 25 healthy food-handlers (18%). With the HGMF-CH method, *cpe*-positive *C. perfringens* type A was isolated from 11 of the 23 persons studied. The average number of *cpe*-positive *C. perfringens* type A and total *C. perfringens* in these samples were 2.5×10^2 CFU/g and 1.8×10^4 CFU/g, respectively. In 10 of 11 individuals, *cpe*-negative *C. perfringens* type A formed a 10- to 1000-fold majority in the *C. perfringens* population. Only *cpe*-positive *C. perfringens* type A isolates were recovered from one person.

The prevalence of different genotypes of *cpe*-positive *C. perfringens* type A was for plasmid-borne IS*1151*-*cpe*, plasmid-borne IS*1470*-like-*cpe*, and chromosomal IS1470-*cpe* 3.7%, 2.9%, and 0.7%, respectively. Previously unknown genotypes were also identified, with a prevalence of 1.5%. In PFGE analysis, from one to five *C. perfringens* isolates with different MRPs were found in each person. Three persons carried *cpe*-positive isolates with two different MRPs. In one case, two *C. perfringens* isolates from the same person shared the same MRP, but showed different *cpe* status; one lacked *cpe* and the other represented plasmid-borne IS*1470*-like-*cpe*. When we tested a group of isolates from each MRP, CPE production was evident in 89%.

5.3 Involvement of different genotypes of *cpe*-positive *Clostridium perfringens* type A in food poisoning outbreaks (V)

All 53 *C. perfringens* isolates from different food poisoning outbreaks were of type A, and 48 (91%) carried *cpe*. Relative prevalences of different genotypes in the examined outbreaks were 75% for chromosomal IS*1470*-*cpe*, 21% for plasmid-borne IS*1470*-like-*cpe*, and 4% for plasmid-borne IS*1511*-*cpe*. Sporulation was successful with 34 (70%) of the investigated isolates, and all of these isolates produced CPE. A total of 31 different PFGE patterns were observed.

6. DISCUSSION

6.1 Development of methods and their suitability for the diagnostics and epidemiology of *cpe*-positive *Clostridium perfringens* (I-III)

The HGMF-CH method provides an effective tool in the investigation of *cpe*-positive *C. perfringens* from feces (I). Prior to the development of this method, several detection methods for *cpe* had been reported, but none facilitated the isolation of *cpe*-positive *C. perfringens*. The main advantage of HGMF-CH is that it allows detection and isolation of *cpe*-carrying colonies among large numbers of *cpe*-negative *C. perfringens*. Thus, the method is unaffected by the presence of vast numbers of *cpe*-negative *C. perfringens* in the samples, which is encountered as a frequent problem when screening *cpe*-positive *C. perfringens* from nature (McClane 2001). The successful use of HGMF-CH in Study IV proved that the method is adequate for the detection and isolation of *cpe*-positive *C. perfringens* from fecal samples. The HGMF-CH method proved invaluable in the isolation of *cpe*-positive strains since in all instances when both *cpe*-positive and *cpe*-negative *C. perfringens* were isolated, the former existed as a minority and thus would have been missed by using conventional isolation methods. By initially screening the samples for the presence of *cpe* and by choosing the PCR-positive samples for HGMF-CH, the costs are reduced and the method is rendered more effective.

When developing the method, occasional filtration problems were encountered. Obstruction of the filtration apparatus and the membrane could be further avoided by using some sample preparation methods prior to filtration. Moreover, diarrheic fecal specimens penetrated the filtration apparatus and the filter better than feces of normal consistency. Thus, in addition to epidemiological studies, the HGMF-CH method seems to be well suited for the diagnostics of diarrheic samples from enteric diseases caused by *cpe*-positive *C. perfringens*.

The multiplex PCR established was found to be a suitable tool for toxinotyping *C. perfringens* isolates and for detecting the presence of *cpe* in these isolates (II). In addition to simultaneous detection of different toxin genes, CPA primers may also be employed in a single PCR assay using the same amplification conditions. Since CPA is produced by each *C. perfringens* type

(A–E), detecting *cpa* gene in a single PCR assay could serve as a rapid screening method to identify *C. perfringens* among other bacterial species. The use of CPA primers in a single PCR reaction would thus be reasonable when aiming to reduce costs and confirming an isolate at the species level.

The AFLP method proved suitable in differentiating *C. perfringens* at the species level as well as in typing *C. perfringens* isolates at the strain level. AFLP was also highly reproducible, relatively fast and easy to use. Moreover, it can be completed within two working days. AFLP can serve in the generation of identification libraries. Intriguingly, the method seemed to overcome the occasional problem of extracellular DNase production encountered when typing *C. perfringens* by other typing methods, such as PFGE. A drawback of the study was that AFLP was unsuitable for differentiating between various toxinotypes (A-E) of *C. perfringens*. However, this is not surprising in view of most of the major toxin genes being located on extrachromosomal elements and thus potentially being movable. Therefore, the toxinotype change of a *C. perfringens* isolate is possible with the loss or acquisition of these extrachromosomal elements. Finally, our finding of *C. perfringens* isolates originating from the same outbreak showing identical fingerprinting patterns indicates that AFLP is a highly discriminative genotyping method and serves as an attractive alternative to PFGE typing in epidemiological studies.

6.2 Epidemiology of *cpe*-positive *Clostridium perfringens* type A (IV and V)

6.2.1 Healthy food-handler as a reservoir for *cpe*-positive *Clostridium perfringens* type A (IV)

When investigating the presence of *cpe*-positive *C. perfringens* in the feces of healthy food-handlers, the organism was found to be widely distributed in this ecologic niche, being carried by 18% of the population studied. By using the HGMF-CH method, we demonstrated that *cpe*-positive strains are frequently present in human feces in low numbers among dominant *cpe*-negative *C. perfringens*. The study showed that healthy humans are a rich reservoir for *cpe*-positive *C. perfringens* type A. The presence of strains representing all identified genotypes (plasmid-borne IS*1151*-*cpe*, plasmid-borne IS*1470*-like-*cpe*, and chromosomal

IS*1470-cpe*) as well as strains carrying previously unrecognized genetic arrangements attached to *cpe* reveals that various different subpopulations of *cpe*-positive *C. perfringens* type A occur in the human GI tract. This also shows that *cpe* is widely dispersed in *C. perfringens* strains belonging to human GI microbiota, indicating that CPE production is an advantageous trait for *C. perfringens* in this niche. *cpe* may be stored in strains kept in low numbers and then laterally transferred to other *C. perfringens* strains when conditions are favorable.

The common occurrence of *cpe*-positive *C. perfringens* type A in the feces of healthy food-handlers and the full capacity of these strains to produce CPE indicate that humans handling food should be considered a possible source of food contamination. If the low numbers of *cpe*-positive *C. perfringens* type A strains are transferred to the food and favorable conditions occur, strains may multiply in the food and cause food poisoning. The person handling food should thus be regarded as a risk factor for spread of *cpe*-positive *C. perfringens* type A food contamination.

6.2.2 Different genotypes of *cpe*-positive *Clostridium perfringens* type A in food poisoning outbreaks (V)

Chromosomal *cpe*-carrying *C. perfringens* type A isolates have been considered to be the causative agent in *C. perfringens* type A food poisonings, whereas *C. perfringens* type A strains carrying plasmid-borne *cpe* have been linked with other CPE-associated diarrheas. Interestingly, in only 75% of the Finnish and German food poisoning outbreaks, chromosomal *cpe*-carrying *C. perfringens* type A was the causative agent. Thus, *C. perfringens* type A carrying plasmid-borne *cpe* was involved in 25% of these food poisonings. Previously, plasmid-borne *cpe* strains have been considered atypical causes of food poisonings (Tanaka *et al*. 2003, Nakamura *et al*. 2004). These results offer the new data that plasmid-borne *cpe* strains cause a notable proportion of *C. perfringens* type A food poisoning outbreaks in Europe. A recent report suggests that this also seems to be the case in Japan (Tanaka *et al*. 2007). Thus, emerging evidence shows that plasmid-borne *cpe* strains are causing food poisonings globally, and that the generally accepted relationship between *cpe* genotype and CPE-associated GI disease is not straightforward. Although chromosomal *cpe*-carrying *C. perfringens* type A strains seem to be the major cause of *C. perfringens* type A food

poisoning, plasmid-borne *cpe*-carrying *C. perfringens* type A strains must also be taken into account as an important agent causing this common food poisoning.

It seems also obvious that CPE-associated GI diseases may not be strictly divided into foodborne and non-foodborne groups. Antibiotic-associated diarrheas and sporadic diarrheas with the causative agent being plasmid-borne *cpe*-carrying *C. perfringens* type A have previously been termed "non-foodborne CPE-associated diseases" (Collie and McClane 1998, Collie *et al*. 1998, Sparks *et al*. 2001). Regarding our findings that plasmid-borne *cpe*-carrying strains cause a notable proportion of *C. perfringens* type A food poisoning outbreaks, these strains appear to frequently be transferred to the human GI tract with food. Therefore, in some antibiotic-associated diarrhea and sporadic diarrhea cases, the origin of the causative agent may have been food. Alternatively some of the sporadic diarrheas caused by *cpe*-positive *C. perfringens* type A may have actually been "classical" food poisonings, where large numbers of *cpe*-positive *C. perfringens* cells are ingested, resulting in CPE production and diarrheic symptoms. Regarding the results of our studies, we consider it questionable to assess whether diarrhea caused by plasmid-borne *cpe*-carrying *C. perfringens* type A is truly foodborne or not unless epidemiological studies are conducted.

6.2.3 Epidemiology of *cpe*-positive *Clostridium perfringens* type A (IV and V)

Chromosomal *cpe*-carrying strains cause the majority of food poisoning outbreaks worldwide. These strains have been shown to possess exceptional resistance to heat, cold, and other stresses present in the food processing environment, such as high salt and nitrite concentration (Li and McClane 2006). Chromosomal *cpe*-carrying strains may thus harbor a specific niche in the food processing environment, which is also evidenced by the study demonstrating their predominance in cold-stored retail foods (Wen and McClane 2004). The survival and circulation of these strains may therefore be premised on the survival of vegetative cells and spores in foods and in the kitchen environment.

The presence of the chromosomal *cpe*-carrying strains in the GI tract of healthy humans indicates that these strains are frequently present in our environment and also that healthy persons handling food may transfer these strains into foods. However, not much is known

about what happens to chromosomal *cpe*-carrying strains when they are defecated and thus spread in copious amounts in the environment by the diarrheic person suffering from *C. perfringens* type A food poisoning. The environmental routes of these strains causing food poisonings remain to be elucidated. Considering that chromosomal *cpe*-carrying strains survive in colder temperature better than plasmid-borne *cpe*-carrying strains (Li and McClane 2006), they might also be able to survive better in sewage conditions and represent predominance in that environment compared with plasmid-borne *cpe*-carrying strains. If these chromosomal *cpe*-carrying strains can survive under the extreme conditions in sewage, they could circulate back to the kitchen via environmental contamination or via drinking water.

In support of the theory that chromosomal *cpe*-carrying strains harbor an environmental reservoir is that they have not been related to any diseases other than food poisonings. *C. perfringens* type A food poisoning is thought to be caused by a large bolus of *cpe*-positive *C. perfringens* type A cells that are perhaps subsequently flushed away (Smedley *et al*. 2004). No evidence exists that chromosomal *cpe*-carrying strains would are capable of causing diseases endogenously in the human GI tract. Moreover, it seems that they could possess an environmental reservoir, and by multiplying in the food and producing CPE resulting in diarrhea, they would be effectively dispersed in the environment again. Survival in the environment would then facilitate these strains gaining re-access to the food chain.

The epidemiology of plasmid-borne *cpe*-carrying strains may well differ from that of chromosomal *cpe*-carrying strains. If the chromosomal *cpe*-carrying strains have an environmental reservoir, plasmid-borne *cpe*-carrying *C. perfringens* type A strains may be adapted to the intestinal niche. Plasmid-borne *cpe*-carrying *C. perfringens* type A strains have been linked to antibiotic-associated diarrhea and sporadic diarrhea as well as to animal diarrheas (Cornillot *et al*. 1995, Katayama *et al*. 1996, Collie and McClane 1998, Sparks *et al*. 2001). Our findings of plasmid-borne *cpe*-carrying strains being a common causative agent also in food poisonings reveal this subgroup of *cpe*-positive *C. perfringens* type A to have an extremely wide disease repertoire and to possess a major pathogen. In early studies, these strains have been referred to more frequently than chromosomal strains in nature (Katayama *et al*. 1996). This may well hold true when considering the wide disease repertoire they cause.

The theory about plasmid-borne *cpe*-carrying *C. perfringens* type A strains being adapted to the human GI microbiota and chromosomal *cpe*-carrying strains being environmental strains

is strengthened by several findings. Plasmid-borne *cpe*-carrying *C. perfringens* type A strains have been found to contain also other plasmids, whereas the carriage of plasmids by chromosomal *cpe*-carrying strains is not considered common (Raju and Sarker 2005). In order to survive in the gut environment, microbes face great demands to adapt to the host immunology and to compete with other organisms. Plasmids are known to carry various genes that, for example, assist in rapid colonization in different hosts. Thus, by the means of plasmid acquisition, the plasmid-carrying strains may be better adapted and diversified in different ecological niches (Daubin *et al*. 2003). By contrast, the chromosomal *cpe*-carrying *C. perfringens* strain SM101 has been observed to harbor sequences similar to bacteriocin gene clusters; the finding suggested to represent an adaptation for competition and survival in complex microbial communities such as in sewage or soil (Myers *et al*. 2006).

As mentioned earlier, *C. perfringens* type A food poisoning is typically considered to be caused by ingestion of a large bolus of *cpe*-positive *C. perfringens* type A cells, being subsequently flushed away, with symptoms normally limited to 24 hours. On the contrary, antibiotic-associated diarrhea and sporadic diarrhea caused by plasmid-borne *cpe*-carrying *C. perfringens* type A strains is thought to be caused by low numbers of ingested cells, with the diarrheic symptoms differing from that of *C. perfringens* type A food poisoning by their severity and longevity. Older age is a risk factor for antibiotic-associated diarrhea caused by *cpe*-positive *C. perfringens* type A (Asha *et al*. 2006). Considering our finding of plasmid-borne *cpe*-carrying strains also being capable of causing food poisonings, it would be interesting to determine whether the infectious dose, symptomology, and other characteristics of the disease are similar in *C. perfringens* type A food poisoning caused by chromosomal and plasmid-borne *cpe*-carrying strains. Whether elderly people are more susceptible to the food poisoning caused by plasmid-borne *cpe*-carrying strains should also be clarified. As *C. perfringens* type A food poisonings often occur in institutionalized settings, which often involve elderly people, it would thus be understandable that these places commonly possess *C. perfringens* type A food poisonings (Smith 1998). Future studies remain to be conducted to unravel the epidemiology and routes of different genotypes of *cpe*-positive *C. perfringens* type A in nature. Determining reservoirs of these strains facilitate understanding of the movements of these strains and allows better control and prevention of diseases caused by *cpe*-positive *C. perfringens* type A.

The theory of the movable nature of *cpe* is further strenghtened by the results of Study IV, since multiple strains carrying the same *cpe* plasmid, as well as isolates possessing the same PFGE pattern but different *cpe* status were found from one individual. The findings indicate a loss or acquisition of the *cpe* plasmid by *C. perfringens* strains. In conclusion, these findings offer evidence of *in vivo* horizontal transfer of *cpe* between *C. perfringens* strains in the human GI tract. They also support the theory that AAD or SD caused by *cpe*-positive *C. perfringens* type A may occur as an endogenous infection, the organism being present in the GI tract and thus being capable of causing diseases after exposure to antimicrobial drugs or other predisposing factors.

7. CONCLUSIONS

1. The HGMF-CH method provides a marked improvement in the investigation of *cpe*-positive *C. perfringens* from feces. The method allows the detection and isolation of *cpe*-carrying colonies among large numbers of bacterial growth, and is thus unaffected by the presence of numerous *cpe*-negative *C. perfringens* in the samples, which is often a problem when screening *cpe*-positive *C. perfringens* from nature.

2. The multiplex PCR assay, modified from a previously published protocol, provides a specific tool for toxinotyping and determining the presence of *cpe* from *C. perfringens* isolates.

3. AFLP was suitable for the identification of *C. perfringens* among other *Clostridium* species as well as for typing *C. perfringens* at the strain level. The AFLP protocol was highly reproducible, easy to perform, and relatively fast, enabling high throughput of samples.

4. Although the different genotypes of *cpe*-positive *C. perfringens* type A have been previously characterized and linked to different diseases in several studies, no data have existed about the relative prevalence of these genotypes in different niches, such as in the human GI tract. Our studies showed that the healthy human is a rich reservoir for *cpe*-positive *C. perfringens* type A, carrying all previously characterized genotypes as well as *cpe*-positive *C. perfringens* type A strains with unknown genetic arrangement attached to *cpe*. The findings indicate that humans should be considered a possible source of contamination for *C. perfringens* type A food poisoning. If the low numbers of *cpe*-positive *C. perfringens* type A strains that are present in the human feces are transferred to food and favorable conditions occur, the strains can multiply in the food and cause food poisoning. Persons handling foods should thus be regarded as risk factors for spread of *C. perfringens* type A food poisoning.

The previously proposed theory of the movable nature of *cpe* is strengthened by the results here since multiple strains carrying the same *cpe* plasmid as well as isolates possessing same PFGE pattern but different *cpe* status were found in one individual. An *in vivo* loss or acquisition of the *cpe* plasmid by these *C. perfringens* strains was evident, and the findings therefore support the theory that AAD or SD caused by *cpe*-positive *C. perfringens* type A may occur as an endogenous infection, the organism being present in the GI tract and being capable of transferring the *cpe* plasmid after exposure to predisposing factors.

Plasmid-borne *cpe*-carrying *C. perfringens* type A was shown to cause a notable proportion of *C. perfringens* type A food poisoning outbreaks in Europe. Therefore, the generally accepted relationship between *cpe* genotype and CPE-associated GI disease is not straightforward. In addition to chromosomal *cpe*-carrying *C. perfringens* type A strains, also plasmid-borne *cpe*-carrying *C. perfringens* type A strains have to be taken into account as an important agent causing *C. perfringens* type A food poisoning. The results also indicate that the commonly approved nomination of CPE-associated diseases as non-foodborne and foodborne is by definition unclear. Hitherto, CPE-associated diseases, such as AAD and SD, with the causative agent being plasmid-borne *cpe*-carrying *C. perfringens* type A have been named "non-foodborne CPE-associated diseases". Regarding the findings of this work that plasmid-borne *cpe*-carrying *C. perfringens* type A strains are also frequently transferred to the human GI tract with food, it is questionable whether the diarrhea caused by plasmid-borne *cpe*-carrying *C. perfringens* type A is truly non-foodborne without epidemiological studies.

In conclusion, chromosomal *cpe*-carrying strains may harbor a specific niche in the food processing environment, whereas plasmid-borne *cpe*-carrying *C. perfringens* type A strains could be adapted to the intestines. We showed that both chromosomal and plasmid-borne *cpe*-carrying *C. perfringens* type A strains can cause food poisonings, and both may be present in the feces of healthy humans. Future studies should unravel whether the epidemiology and routes of these different genotypes of *cpe*-positive *C. perfringens* type A differ from each

other. Improved understanding of the movements of these strains would help to better control and prevent associated diseases.

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